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(54) **Protease for activating clotting factor VII**

Protease zum Aktivierung des Gerinnungsfaktor VII

Protéase pour l'activation du facteur de coagulation VII

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(56) References cited:
WO-A-91/01497
WO-A-96/10638
FR-A- 2 504 921

- RÖMISCH J. ET AL.: "A protease isolated from plasma which activates FVII in a tissue factor independent manner but inactivates FV and FVIII" ANNALS OF HEMATOLOGY, vol. 78, no. Suppl.1, 24 - 27 February 1999, page A10 XP001059496
- RÖMISCH J. ET AL: "The FVII activating protease mediates fibrinolytic effects activating single-chain plasminogen activators" ANNALS OF HEMATOLOGY, 24 - 27 February 1999, page A24 XP001059495
- KAZAMA ET AL.: "Hepsin, a putative membrane-associated serine protease, activates human Factor VII and initiates a pathway of blood coagulation on the cell surface leading to thrombin formation" JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 270, no. 1, 6 January 1995 (1995-01-06), pages 66-72, XP002190032
- CHOI-MIURA ET AL.: "Purification and characterization of a novel hyaluronan-binding protein (PHBP) from human plasma: it has three EGF, a Kringle and a serine protease domain, similar to Hepatocyte Growth Factor Activator" JOURNAL OF BIOCHEMISTRY, vol. 119, no. 6, 1996, pages 1157-1165, XP000960750
- HUNFELD A ET AL.: "Identification of the thrombin-like activity of PCCs" ANNALS OF HEMATOLOGY, vol. 76, no. Suppl.1, 25 - 28 February 1998, page A101 XP001059492
- LAAKE K & OSTERUD B: "Activation of purified plasma factor VII by human plasmin, plasma kallikrein and activated components of the human intrinsic blood coagulation system" THROMBOSIS RESEARCH, vol. 5, no. 6, December 1974 (1974-12), pages 759-772, XP008000261
- HUNFELD A. ET AL: 'Detection of a novel plasma serine protease during purification of vitamin K-dependent coagulation factors' FEBS LETTERS vol. 456, 1999, pages 290 - 294
- RÖMISCH J. ET AL: 'A protease isolated from human plasma activating Factor VII independent of tissue factor' BLOOD COAGULATION AND FIBRINOLYSIS vol. 10, no. 8, 1999, pages 471 - 479

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Description

[0001] The invention relates to a protease for activating the blood clotting factor VII, to a process for isolating it, detecting it and inactivating it, and to medicinal preparations which comprise this protease.

[0002] The blood clotting system comprises two different, cascade-like pathways for activating clotting factors which are present in the plasma. The intrinsic or the extrinsic pathway is preferentially used for initiating clotting, depending on the triggering mechanism.

[0003] When a tissue is damaged, thromboplastin (tissue factor, TF with phospholipids) is exposed by the affected cells as the starter of the extrinsic clotting pathway. The membrane-located thromboplastin can bind both clotting factor VII (FVII) and circulating, activated FVII (FVIIa). In the presence of calcium ions and lipids, this TF-FVIIa complex leads to the binding of FX, which is converted into its activated form (FXa) by limited proteolysis. FXa in turn leads, by activating prothrombin to form thrombin, to the formation of fibrin and thereby ultimately to closure of the wound.

[0004] While the further activation of the thromboplastin-bound FVII initially takes place autocatalytically, in particular, it is supported, after the clotting cascade has been initiated, by FXa and thrombin, in particular, leading to marked reinforcement of the reaction cascade.

[0005] The administration of FVIIa or FVIIa-containing concentrates is indicated in certain clinical situations. The so-called FVIII-bypassing activity (FEIBA) of FVIIa is used in patients who are suffering, for example, from hemophilia A and have developed antibodies against FVIII as a consequence of the administration of FVIII. According to presently available findings, FVIIa is well tolerated in this context and, while it does not lead to any tendency to thrombosis, it is suitable for ensuring that clotting takes place to a limited but adequate extent Recombinant FVIIa is already being used therapeutically and prophylactically. FVII which has been isolated from blood plasma can also be activated and then used. Proteases such as thrombin can be used for this activation; however, these proteases, as such, can themselves strongly activate clotting and lead to the risk of a thrombosis. For this reason, subsequent removal or inactivation of thrombin is necessary and leads to yield losses. As a result of the risk of thrombosis which is associated with it, the use of FXa or FIIa (thrombin) is frequently contraindicated and only indicated in emergencies, e.g. in association with extreme loss of blood and unstaunchable hemorrhages.

[0006] FVIIa is found in very low concentrations in the plasma of healthy subjects. Only very little is so far known about the formation and origin of FVIIa which is circulating in the blood. Traces of thromboplastin which has been expressed or released in association with cell destruction might play a role in this context. Although it is known that factor XIIa, for example, can lead to FVII activation under certain conditions, the physiological relevance of this reaction has not yet been clarified.

[0007] Surprisingly, a FVII-activating protease, which differs from all the previously known proteases, has now been found in connection with fractionating human plasma and certain prothrombin complex concentrates. Investigations into this protease have shown that it exhibits a particularly high amidolytic activity toward the peptide substrate S2288 (HD-isoleucyl-L-protyl-L-arginine-pNA) from Chromogenix AB, Sweden. A particular feature of this protease is that the amidolytic activity is efficiently inhibited by aprotinin. Other inhibitors, such as the antithrombin III/heparin complex, are also suitable for the inhibition. On the other hand, its activity is increased by heparin and heparin-related substances such as heparan sulfate or dextran sulfate and calcium ions. Finally, it has been found that this protease is able, in a manner dependent on time and on its concentration, to convert FVII into FVIIa. This reaction, too, is inhibited by aprotinin.

[0008] Part of the subject matter of the invention is therefore a protease for activating the blood clotting factor VII, which

a) is inhibited by the presence of aprotinin,

b) is increased in its activity by calcium ions and/or heparin or heparin-related substances, and

c) in SDS-PAGE, on subsequent staining in the non-reduced state, has one or more bands in the molecular weight range from 50 to 75 kDa and kDa in the reduced state has a band at 40 to 55 kDa and one or more bands in the molecular weight range from 10 to 35 kDa.

[0009] In the following text, the activated form of the protease is termed "protease" whereas the non-activated form is termed "proenzyme".

[0010] Further investigations with this protease have shown that, after enriching or isolation, it suffers from a rapid loss of activity, which was observed in a solution containing 20 mM tris, 0.15 M NaCl at a pH of 7.5. The addition of albumin at a concentration of 0.1 % was not able to prevent the activity of the protease from decreasing by 50% after one hour at room temperature. On the other hand, very good stabilization of the protease was observed in a solution which was buffered to a pH of 6.5 with 50 mM Na citrate. If no particular stabilizers are added to the protease solution, no, or only slight, losses in activity are observed if the solution is adjusted to a pH of between 4 and 7.2. preferably to a pH of between 5.0 and 7.0. However, it is expedient to add stabilizers to the solution, with suitable stabilizers, apart

from citrate, being, in particular, glutamate, amino acids, such as arginine, glycine or lysine, calcium ions and sugars such as glucose, arabinose or mannose in quantities of 1-200 mmol/l, preferably in quantities of 5-100 mmol/l. Efficient stabilization was also achieved by adding glycols such as ethylene glycol or glycerol, with quantities of 5-80% by weight, preferably of 10-60% by weight, being used. The pH of the stabilized solution should then be between the pH values 4-9.

[0011] The novel protease, and also the proenzyme, can be obtained by fractionation of blood plasma or of prothrombin complex (PPSB) concentrates. The starting material is then first of all subjected to an anion exchange chromatography, which is followed by an affinity chromatography of the eluate. A heparin which is immobilized on a matrix, or a heparin-related substance such as heparan sulfate or dextran sulfate, is particularly suitable for the affinity chromatography. When such a chromatographic method is used, the novel protease and/or the proenzyme can be selectively bound and then eluted once again using known methods. The use of a spacer is advisable for coupling the ligand to the matrix. A heparin-lysine matrix has been found to be particularly suitable for isolating the novel protease.

[0012] In SDS-PAGE with subsequent staining, the protease which has been isolated by this method exhibits, in the non-reduced state, one to several bands which lie closely together in the molecular weight range of 55-75 kDa. Following reduction, one to several bands were observed in the molecular weight range of 15-35 kDa and one band was observed at 40-55 kDa. A further band between 60 and 65 kDa, which, after scanning and quantitative evaluation, constituted 5-10% of the total protein, showed that non-activated proenzyme was also present. This result was supported by appropriate investigations using monoclonal antibodies against this protease. It was therefore concluded that the proenzyme of this protease can also be prepared, pasteurized and used by the method according to the invention. Part of the subject matter of the invention is therefore the proenzyme of the protease for activating blood clotting factor VII. The proportion of the proenzyme is indicated by the band between 60 and 65 kDa. Corresponding to the amino acid sequence which constitutes the activation region of the proenzyme, thrombin, kallikrein or FXIIa are, in accordance with their substrate specificities, examples of suitable physiological activators of the proenzyme.

[0013] Some of the properties of the novel protease which have been described, namely the fact that it can be isolated from plasma or from prothrombin complex (PPSB) concentrates which are derived from plasma, the inhibition of its amidolytic activity by aprotinin and the described migration behavior in SDS-PAGE, both in the reduced and in the non-reduced states, are reminiscent of a protease which was isolated by Hunfeld et al. (Ann. Hematol. 1997; 74; A87, 113; Ann. Hematol. 1998; 76; A101, P294 and Etscheid et al. Ann. Hematol. 1999, 78: A42) from a PPSB concentrate which was not defined in any more detail. In that case, the preparation was essentially achieved using an aprotinin matrix. As a result of the amidolytic cleavage of certain peptide substrates, the activity was described as being a thrombin-like activity. Hunfeld et al. did not find any influence on global clotting parameters such as prothrombin time, Quick or platelet aggregation.

[0014] The N-terminal sequencing of the protease described by Hunfeld et al. shows concordances with a protein whose cDNA was described by Choi-Miura et al. (J. Biochem. 119: 1157-1165 (1996)). In its primary structure, the corresponding protein exhibits homology with an enzyme termed hepatocyte growth factor activating enzyme (HGFA).

[0015] When two bands which were isolated from SDS-PAGE under reducing conditions were subjected to N-terminal sequencing, the following concordances were established:

Molecular weight range of the band	Amino acid sequence	Author
10-35 kDa	IYGGFKSTAGK	present invention
30 kDa	IYGGFKSTAG	Hunfeld et al.
17 kDa	IYGGFKSTAGKH	Choi-Miura et al.
40-55 kDa	LLESLDP	present invention
50 kDa	SLDP	Hunfeld et al.
50 kDa	SLLESLDPWTPD	Choi-Miura et al.

[0016] Concordances are also found in other test results such as substrate specificity and the ability of the activity to be inhibited. Despite this, it is still not possible at present to assume with confidence that these proteins are identical. At any rate, the previously investigated, abovementioned proteins have not been reported to possess the property of activating FVII or activating other factors (see below).

[0017] On the basis of its described properties, the novel protease can be used diagnostically and therapeutically.

1. Test systems using the novel protease

[0018] The novel protease can be used diagnostically in test reagents. Thus, the presence of factor VII can be deter-

mined qualitatively and quantitatively in a clotting test by adding the novel protease.

[0019] Conversely, the test system developed for measuring FVII activation can also be used for detecting and quantifying the protease. For this, a solution containing the protease is mixed with an FVII-containing solution and, after an appropriate incubation time, the resulting quantity of FVIIa is quantified. This can be carried out, for example, using the Staclot® FVIIa-rTF test (Stago/Boehringer Mannheim). When a preferred procedure is used, this test is not limited by the FVII concentration supplied. If the quantity of protease in the form of the proportion of total protein is known, which proportion can be determined

- in a pure protease preparation, by means of the Kjeldahl method or by means of another protein assay with which the skilled person is familiar, or
- using an antigen test, for example based on specific antibodies and an appropriate immunochemical determination method such as ELISA, the specific activity of the protease preparation can then be measured in a corresponding manner.

[0020] Surprisingly, a property has now been found, in association with characterizing the protease further, which makes it possible to carry out an additional determination method. In association with incubation of the blood clotting factors VIII/VIIIa and V/Va with said protease, and subsequent quantification, it became clear that said clotting factors are inactivated in a manner which is dependent on the protease concentration and on the length of the incubation.

[0021] Another part of the subject matter of the invention is therefore a novel test system for qualitatively and quantitatively detecting the protease which activates blood clotting factor VII, in which system the protease can be determined by its action inactivating the blood clotting factors VIII/VIIIa or VNa. This test system is based on a solution containing the protease being incubated with factor VIII/VIIIa or factor V/Va and the remaining quantity of factor VIII/VIIIa or the remaining quantity of factor V/Va being measured by means of a conventional activity test and the amount of protease then being quantitatively determined from this by comparison with a standard curve. In carrying out this test, the incubation of the protease activity is inhibited, after predetermined periods of time, by the limited addition of aprotinin, which has the advantage that it has no effect, at these concentrations, on the subsequent measurements of the test system. After that, the remaining activities of the clotting factors are measured by means of a test which is familiar to the skilled person. For this, a test system has, in particular, proved its worth in which use is made of the so-called Coamatic® factor VIII test (Chromogenix AB), which essentially contains factors IXa and X, with the resulting amount of FXa being quantified, in the presence of a thrombin inhibitor, by means of the conversion of a chromogenic substrate (see last third of page 2). This amount is in turn proportional to the quantity of FVIII or FVIIIa. Determining the residual FVIII activity then makes it possible to deduce the concentration of protease which is present.

[0022] The degradation of the FVIII/FVIIIa or the FV/FVa due to the proteolytic effect can be clearly demonstrated by SDS-PAGE. Depending on the time for which the protease is incubated, for example, with an FVIII concentrate, bands which are typical for FVIII disappear while other, new bands emerge or weak bands increase in intensity. Accordingly, the activity of the protease can also be correlated by quantifying the decreasing or increasing bands and consequently measured quantitatively, for example using a protease standard. The changes in the band intensities on the SDS-PAGE electropherogram or following other electrophoretic methods can be quantified, for example, using a scanner, with which a skilled person is familiar, and the appropriate program. In addition to this, antibodies against said clotting factors can be used for Western blotting and employed for evaluation in the manner described. Antibodies which specifically detect the decreasing bands or, in particular, the emerging bands are particularly suitable. In this context, these antibodies can also be used for establishing other immunochemical tests such as an ELISA.

[0023] The proteolytic inactivation which has been described in the case of FVIII/FVIIIa is also observed when the protease is incubated with factor V/Va, which exhibits a certain degree of structural homology with FVIII. The degradation can be monitored in suitable activity test systems and in SDS-PAGE/Western blotting.

[0024] Despite the inactivations of FV and FVIII, it was now found that adding the protease to blood, to platelet-rich plasma or plasma shortened the clotting times, that is the procoagulatory effect predominated in various so-called "global clotting tests". These test systems are understood as being, for example, the non-activated partial thromboplastin time (NAPTT), the prothrombin time (PT) and the recalcification time. Since the shortening of these times, as measured, for example, in so-called coagulometers, by means of thromboelastography or else in chromogenic tests, correlates with the concentration of a clotting-promoting substance, the concentration of the substance in a sample can conversely be deduced using a calibration curve of the clotting time. The concentration of the "FVII activator" can correspondingly be determined using selected global clotting tests.

[0025] It was also surprising to find that the "FVII activator" is likewise able to bring about effective activation of single chain urokinase (scuPA, single chain urokinase plasminogen activator) and single chain tPA (sctPA, single chain tissue plasminogen activator), that is can act as a plasminogen activator activator (PAA). The activity of the activated PAs can be measured, for example, using chromogenic substrates. Accordingly, this property can therefore also be used for

detecting and quantifying the "FVII activator". The activation of the plasminogen activators can also be determined in a coupled reaction in the presence of plasminogen, either by the formation of plasmin itself or by the dissolution of a fibrin clot which is brought about by plasmin.

[0026] In summary, therefore, it can be stated that the protease can be both detected and quantified by incubating it with a solution containing FVIII or FVIIIa and then determining the remaining quantity of FVIII/VIIIa by means of a suitable activity test. In the same way, FV or FVa can be incubated with the protease and the remaining quantity of FV/FVa can subsequently be quantified. The unknown protease concentration can be determined quantitatively by comparison with a standard curve of increasing quantities of protease which is included in the test. Various global clotting tests are likewise suitable for the quantification, with the protease concentration being read off a calibration curve on the basis of the shortening of the clotting time. The PAA activity of the protease can also be used for determination purposes.

[0027] Another feature of these tests is that the FV and FVIII inactivation and the PAA activity are displayed particularly well in the presence of adequately high concentrations of calcium, preferably > 0.001 mM, particularly Preferably > 0.005 mM, e.g. in the form of $CaCl_2$. In contrast to the direct chromogenic assay, in which, as has been described above, both heparin and heparin-like substances and also calcium increase the protease activity, the inactivation of FV/FVIII is not promoted, or only promoted insignificantly, by heparin. By contrast, the PAA activity is stimulated in the presence of both agents, that is by calcium and/or heparin or heparin-like substances.

[0028] The protease-mediated reactions can be very efficiently diminished or prevented by incubating the protease with inhibitors, particularly antithrombin III in the presence of heparin or heparin-like substances (preferably in the presence of heparin), C1-esterase inhibitor, alpha2-antiplasmin, inter-alpha-trypsin inhibitor or known synthetic, low molecular weight protease inhibitors such as Guanidinocaproic acid-para-ethoxycarbonylphenylester wich is available under the trademark FOY®. These substances can therefore be used for stopping the reaction, in order, for example, to define incubation times precisely or to increase the specificity of the test still further. Decreasing the free calcium ions in the mixture with a chelating agent, for example, can also be used for this purpose.

2. Stabilized preparations of factor V and factor VIII

[0029] The further task now ensued, from the above-described observations concerning the proteolytic actions of the novel protease on clotting factors V and VIII, of inhibiting the protease or reducing its activity in order to avoid losses of yield and the formation of what might possibly be interfering protein fragments. This is all the more relevant since FV and FVIII are usually prepared from cryoprecipitates which have been obtained from plasma and in the presence of calcium ions because the latter are required for maintaining protein conformations.

[0030] Another part of the subject matter of the invention is therefore a stabilized preparation of FV or FVIII which is free of the factor V or factor VIII fragments formed due to proteolytic degradation as a result of the fact that the protease activating the blood clotting factor VII is inhibited. Since more detailed investigations have shown that inactivation of factor V and factor VIII by said protease takes place particularly efficiently in the presence of calcium ion concentrations greater than 0.5 mM, the factor V or VIII preparation can be effectively stabilized if, for the inhibition of the protease activating the blood clotting factor VII, the concentrations of calcium ions in the factor V or in the factor VIII preparation are adjusted to less than 1.0 mM, preferably to less than 0.5 mM. While the factor V- and factor VIII-inactivating properties of the protease are markedly reduced at these concentrations, the quantity of calcium ions is still sufficient for stabilizing the conformations of the FV and FVIII molecules. The abovementioned quantities of calcium ions should not be exceeded, not merely in the end product but also in the cryoprecipitate itself and in the following purification steps.

[0031] In accordance with the above-described affinity of the protease or the proenzyme for heparin and heparin-like substances, the protease/the proenzyme can be removed from the FVIII- or FV-containing solution by incubating with immobilized heparin or other suitable immune- or affinity-adsorbents. Polyclonal or monoclonal antibodies, respective antibody-fragments that are useful in preparing the immune adsorbents are readily available by techniques known in the art in using all or part of the protease or proenzyme as antigen.

[0032] However, natural or synthetic protease inhibitors can also be employed, where appropriate in addition to decreasing the quantity of calcium ions, for preventing the proteolytic degradation of the FV or the FVIII. Proteins such as aprotinin, alpha2-antiplasmin, C1-esterase inhibitor or inter-trypsin inhibitor may be employed as inhibitors. Low molecular weight substances which are known to the skilled person as synthetic serine protease inhibitors can also be used in this context. Inhibitors, such as antithrombin III, whose inhibitory potential is increased by heparin or heparinoids can likewise be added. Thus, it has been found, surprisingly, that while heparin on its own is able to increase the amidolytic activity of the protease towards small chromogenic substances, it does not support inactivation of FV/FVIII.

3. Pharmaceuticals which comprise the novel protease

[0033] The novel protease and/or its proenzyme can also be used therapeutically.

[0034] They can be employed as a blood coagulating agent, either on their own or together with substances which

increase the activity of the protease, such as heparin, or heparin-related substances, such as heparan sulfate, and/or calcium ions, with it being possible additionally to add factor VII as well, in its inactive form, to this agent. The use of such an agent, in which its FVIII-bypassing activity (FEIBA) is exploited, for example, can be indicated when intolerances exist toward FVIII and/or FIX and/or FXI and/or the contact phase proteins, such as FXII, for example on account of the presence of antibodies, or when other types of deficiency situation exist. In this connection, the FVII can be activated either in vitro, in the plasma, in enriched fractions or by acting on purified FVII. It is also possible to use the novel blood coagulating agent ex vivo for general hemorrhage prophylaxis or for staunching hemorrhages.

[0035] On the other hand, the observed inhibition of the novel protease by aprotinin or the abovementioned inhibitors can be used for developing an agent which comprises a protease inhibitor and which diminishes the ability of the blood to coagulate. In addition to this, the novel protease can also be used to identify physiological or non-physiological factors, such as synthetic peptides, which impair blood clotting because of their protease-inhibiting effect. The peptide sequences of the chromogenic substrates which are transformed particularly efficiently, such as those of the S 2288 (see above for details), can be used as a structural basis for this. The addition of suitable inhibitors to clotting preparations, or during their preparation, can be necessary if these preparations are to be free of proteolytic activities.

[0036] Surprisingly, a property has now been found, in association with characterizing the protease further, which opens up the possibility of an additional use for the so-called "factor VII activator" protease. When single chain plasminogen activators such as prourokinase (single chain urokinase, scuPA, single chain urokinase plasminogen activator) or sctPA (single chain tissue plasminogen activator) are incubated, the "factor VII activator" brings about activation of these plasminogen activators (PA). In this connection, there is a limited proteolysis of the single chain PAs, resulting in the formation of double chain proteases, which are particularly suitable for activating plasminogen. The resulting plasmin is the effector of fibrinolysis, that is the physiological system which is responsible for dissolving thrombi. PAs, such as prourokinase or tPA, are endogenous proteins which are released when needed and which, as is known, are activated by plasmin or by kallikrein (scuPA). The mechanism by which scuPA is activated in the healthy state has not yet been fully clarified.

[0037] The plasminogen activators are employed therapeutically, as isolated or recombinantly prepared proteins, in pharmaceutical preparations in association with thromboembolic diseases or complications, such as in leg vein thrombosis, cardiac infarction or strokes.

[0038] In accordance with the properties of the "factor VII activator" which have now been found, the latter can be used for in vivo or ex vivo activation of plasminogen activators such as prourokinase or sctPA. This activity can also be applied by using said protease for the prophylaxis or therapy of thromboembolic diseases, specifically in combination with single chain or double chain plasminogen activators or anticoagulants as well. This possible use is not contradictory to the fact that the protease is also able to act in a procoagulatory manner. The question of which of the two reactions predominates is probably resolved by the availability of the physiological substrates. According to the current state of knowledge, factor VII is activated moderately in plasma and continuously maintains a certain concentration of FVIIa in order to be able to counteract immediately any sudden vascular damage. On the other hand, only nanogram quantities of tissue plasminogen activator and urokinase plasminogen activator are present in a milliliter of blood plasma. It is only when fibrin deposition or thrombi occur that there is an increase in the concentration, by secretion or synthesis, of plasminogen activators, which then display their thrombolytic activity by activating plasminogen after they have been activated locally, in particular when bound to the thrombus. When single-chain PAs are present, particularly in a locally restricted manner, their activation might outweigh FVII activation, thereby making it possible to adjust to the physiological situation. Accordingly, this protease might also regulate hemostasis, thereby indicating a replacement with the protease and/or the proenzyme in the case of inborn and acquired deficiency states.

[0039] Another part of the subject matter of the invention is therefore a pharmaceutical preparation which comprises a quantity of the blood clotting factor VII-activating protease and/or its proenzyme form which is sufficient for dissolving fibrin-containing thrombi. This preparation may additionally comprise single chain plasminogen activators (PA) and/or anticoagulants. When the proenzyme is present it is advantageous to comprise a suitable activating agent within or together with the pharmaceutical preparation above.

[0040] Since it has been found that the plasminogen activator-reinforcing effect of the "FVII activator" is particularly promoted by calcium and/or heparin and heparin-like substances such as dextran sulfate, pharmaceutical preparations which additional comprise soluble calcium salts and/or heparin or heparin-like substances may particularly advantageously be employed for dissolving, in accordance with the invention, fibrin-containing thrombi. In this context, the protease/proenzyme can be employed on its own or in combination with single chain or double chain plasminogen activators with or without substances which exhibit particular affinities for the protease and thereby increase its activity as carrier substances for prolonging plasma half life or as mediators to surfaces.

[0041] Pharmaceutical preparations which comprise the blood clotting factor VII-activating protease can, because of its special fibrinolytic effect, be employed for treating diseases which are caused by fibrin-containing thrombi. Fibrinolytic processes are also involved in wound healing processes. In this connection, said protease and/or proenzyme can be administered intravenously or locally, subcutaneously, intradermally or intramuscularly, or else topically in the case of

injuries and wounds, or bound to a suitable carrier matrix. Both protease/proenzyme which has been isolated from body fluids such as blood or plasma and protease/proenzyme which has been prepared recombinantly or transgenically can be employed in this context. The protease/proenzyme is also suitable for use as a component of a so-called fibrin adhesive, which should not then contain any substance, such as aprotinin, which inhibits the protease/proenzyme. In this case, use can be made of the clotting-shortening properties of the protease.

[0042] The protease/proenzyme above may be used for inherited or acquired hemostasis deficiencies, in (diffuse) bleeding occurencies respective thrombosis associated complications. If used to treat bleeding the combination of protease/proenzyme together with F VIII optionally under addition of further clotting factors is advantageous.

4. Process for pasteurizing the FVII-activating protease

[0043] As a protein which has been isolated from human plasma, the novel protease and/or its proenzyme can only be employed as a pharmaceutical preparation if it has previously been subjected to a process for inactivating viruses. The pasteurization process is in particular recognized as being the most important process for inactivating viruses. However, heating at about 60°C for up to 10 hours requires the protein which is to be treated to be of adequate stability. The optimal stabilizers have to be determined separately for each protein and their concentrations have to be optimized.

[0044] In the case of the novel protease and/or its proenzyme, conditions which stabilize the protein in solution, without any pasteurization being performed, have already been mentioned above. In this regard, a slightly acidic pH range has in particular proved to be advantageous. However, when a pasteurization is carried out under these conditions, the novel protease and/or its proenzyme as a rule loses more than 50% of its original activity.

[0045] It has now been found that a pasteurization of a pharmaceutical preparation comprising the novel protease and/or its proenzyme ensures optimal stabilization results if the preparation is prepared

a) in a pH range of from 3.5 to 8.0, preferably in a pH range of from 4.0 to 6.8;
b) in the added presence of one or more amino acids in a quantity of more than 0.01 mol/l, preferably more than 0.05 mol/l; and/or
c) in the added presence of a sugar or of a combination of different sugars having a total concentration of more than 0.05 g/ml, preferably more than 0.2 g/ml; and/or
d) in the added presence of one or more substances which are able to complex calcium ions, such as citrate, oxalate, ethylenediamine tetraacetic acid, etc.

[0046] Additives such as albumin, Haemaccel®, heparin and heparinoids, glycerol, glycol and polyethylene glycol, may also be used separately or mixed together. After the pasteurization has been completed, the sugars, amino acids and other additives which have been added as stabilizers can be decreased, or removed completely from the preparation, using methods with which the skilled person is familiar. The results of the pasteurization processes are given in Examples 12 and 13.

Example 1

[0047] The Staclot® FVIIa-rTF test system (Stago/Boehringer Mannheim) was used for demonstrating activation of FVII by the prepared protease. This detection system is based on the particular property of (recombinant) soluble tissue factor (rTF) which is only able to use the preformed activated FVII (FVIIa) for initiating the extrinsic clotting pathway. In contrast to the situation when complete tissue factor is used, this makes it possible to determine the real content of FVIIa precisely.

[0048] Isolated FVII (Enzyme Research Labs) was used for the activation experiments. This FVII itself contains traces of FVIIa since it is isolated from human plasma. The concentration was adjusted to 0.05 IU of FVII/ml by diluting with buffer. The FVII was incubated at room temperature for 10 min with the test substances and then tested for the true FVIIa content. The FVIIa contents were quantified using a reference curve which was constructed in parallel.

[0049] it was ascertained in preliminary experiments, which are not described here, that while, in the concentration employed, aprotinin completely inhibited the activity of the prepared protease, it had no direct effect on the FVIIa nor any significant effect on the FVIIa-rTF test system.

[0050] The results given below relate in each case to triplicate determinations.

[0051] The following experimental assays were accordingly set up:

1. FVII:

Result: 10 mIU of FVIIa/ml

[0052] Non-activated FVII was used as the control assay. This already contains traces of FVIIa (see above) in the order of magnitude of 10 mIU of FVIIa/ml.

2. FVII + aprotinin:

[0053] In this assay, FVII was incubated in the presence of aprotinin and used in the FVIIa-rTF assay in order to demonstrate that FVIIa itself was not inhibited, and nor was the test affected by the aprotinin employed. This was confirmed (in comparison with assay 1).

3. Protease + FVII (incubation), followed by the addition of aprotinin:

Result: 18 mIU of FVIIa/ml

[0054] In this case, the protease was given time to activate FVIIa. Aprotinin was only added, in order to inhibit the protease, after the 10-minute incubation had taken place. The resulting FVIIa was quantified in the FVIIa-rTF assay. Subtracting the FVIIa base value (assay 1), 8 mIU of FVIIa/ml have therefore been formed by the action of the protease under the chosen conditions.

4. Protease + aprotinin, followed by the addition of FVII

Result: 11 mIU of FVIIa/ml

[0055] In this assay, the protease was inhibited with aprotinin before contact with FVII. Neither the subsequent incubation with FVII, nor the following FVIIa quantification led to any significant increase in the FVIIa content (because of the range of variation in the assay, 11 versus 10 mIU/ml in assay 1 is not to be regarded as being significant).

5. Protease

Result: 0 mIU of FVIIa/ml

[0056] This assay demonstrated that, at the concentration selected, the protease did not itself have any effect on the FVIIa-rTF test system.

[0057] In summary, it follows from the above that

- the described protease activates FVII;
- the activation of FVII by the protease takes place "directly", that is independently of the presence of rTF;
- the activation of FVII can be inhibited by aprotinin; at the concentration selected, aprotinin itself does not have any significant influence on the test system.

Example 2

[0058] This example describes how FVII is activated in a reaction which is dependent on the concentration of the protease and on the time over which the protease is incubated with FVII.

[0059] Test systems and reagents were selected to correspond with the conditions described in Example 1. In a first series of experiments, the initally introduced FVII was preincubated with different dilutions (1:5, 1:10 and 1:20) of the protease-containing solutions (5 min at RT), then treated with aprotinin (to inhibit the protease) and subsequently tested for its content of FVIIa in the FVIIa-rTF assay.

[0060] Once again, the parallel assays, in which the protease had been inhibited by aprotinin before contact with FVII, served as control assays.

[0061] The results are given as activation factors, i.e. correspond to x times the value which was measured in the abovementioned control assay:

Assay	Control
Protease + FVII	Protease + aprotinin
Incubation + Aprotinin	Incubation + FVII

Dilution of the protease solution	Activation factor	
1:5	2.6	1.0
1:10	2.0	1.0
1:20	1.6	1.0

[0062] The activation factor 1:0 of the control assays corresponds to the control, which was additionally included and in which only the test buffer, containing the FVII employed, was treated under identical incubation conditions and tested. That is, no significant activation took place in the control assays.

[0063] It follows from this that FVII is activated by the protease in a manner which is dependent on the concentration of the protease.

[0064] It was similarly demonstrated that, when the concentrations of the coreactants are kept constant, the FVII is activated by the protease in a manner which is dependent on the length of the incubation.
When equal volumes of a solution containing 0.2 IU of FVII/ml and a 1:10-diluted protease solution were incubated together, the following contents of FVIIa were obtained after incubating for the relevant times and subsequently adding aprotinin (in order to stop the activation):

Length of incubation	Activation factor
0 min	1.0
2.5 min	1.3
5.0 min	2.0
10.0 min	2.8
40.0 min	> 3.8

[0065] It follows from this that FVII is activated by the protease in a time-dependent manner.

Example 3

[0066] Using this example, it will be demonstrated that activation of FVII by the protease is increased in the presence of calcium ions and heparin.

[0067] 25 μl of the protease-containing solution were mixed with 50 μl of

- buffer (control)
- 15 mM $CaCl_2$
- 50 USP units of heparin/ml
- Pathromtin (lipid mixture, aliquot dissolved in accordance with the manufacturer's instructions)

at room temperature for 5 min, and then treated with 150 μl of a tris/NaCl buffer solution (pH 8.2) and 25 μl of the chromogenic substrate S2288 (3 mM); the time-dependent change in the extinction at 405 nm was then measured (at 37°C). The activation factors, related to the buffer control (x times), are given in the following table.

Assays	Activation factor (x times buffer control)
Buffer control	1.0
+ $CaCl_2$	3.6
+ Heparin	2.6
+ Lipid	0.9
+ $CaCl_2$ + heparin	4.3

Table continued

Assays	Activation factor (x times buffer control)
+ $CaCl_2$ + lipid	3.3
+ Heparin + lipid	2.7
+ $CaCl_2$ + heparin + lipid	3.7

[0068] Under the conditions used in this example, marked increases in the activity of the protease can be observed in the presence of calcium ions and/or heparin.

Example 4

[0069] In each case, 25 µl of a solution, containing 10, 1 or 0.1 µg of protease/ml were mixed with 25 µl of FVIII (2 IU/ml), after which 25 µl of $CaCl_2$ (25 mM) and 25 µl of Pathromtin® (Dade Behring GmbH) were added. After incubating at 37°C for 0, 3, 10 and 20 min, the reaction was stopped by adding 400 µl of aprotinin (500 KIU/mg). A sample in which aprotinin was introduced initially served as a control.

[0070] Each sample was diluted in tris-buffer/BSA. In each case, 50 µl of this solution were mixed with 50 µl of the factor reagent (essentially composed of FIXa, FX and a thrombin inhibitor, appropriately modified in accordance with the Coamatic® FVIII test, Chromogenix AB) and incubated at 37°C for 10 min. After 50 µl of substrate (e.g. S 2765, N-a-Cbo-D-Arg-Gly-Arg-pNA) had been added, the reaction was stopped after a predetermined period of incubation by adding 50 µl of acetic acid (50%), and the OD405nm was then measured. A standard curve for FVIII was used for determining the concentration in the sample.

Results:

[0071] In a first assay, the time for which the protease was incubated with FVIII (2 IU/ml) was kept constant (10 min) but the concentration of the protease was varied (0.1, 1 and 10 µg/ml). The reaction was stopped and the residual concentration of active FVIII was determined. As the protease concentration increased, correspondingly more FVIII was inactivated (Figure 1).

[0072] The protease content of a sample can be quantified using an appropriate standard curve.

[0073] In a second assay, the concentration of the protease was kept constant (10 µg/ml) but the time of incubation with FVIII (2 IU/ml) was varied. A marked reduction in the residual concentration of active FVIII was seen as the length of incubation increased (Figure 2).

Example 5

[0074] The influence of the "FVII activator" on the activity of factor V was investigated: 25 µl of protease-containing solution (0-100 µg/ml) were incubated with 50 µl of FV (5 IU/ml) and 25 µl of 25 mM $CaCl_2$ (0-20 min) and, after that, 400 µl of buffer containing 100 KIU of aprotinin/ml were added.

[0075] In each case, 100 µl of each incubation assay were then incubated with 100 µl of FV-deficient plasma at 37°C for 1 min, after which 200 µl of Thromborel S® were mixed in and the clotting times were determined in a Schnitger and Gross coagulometer. The residual activities of FV were determined.

Results:

[0076]

Protease concentration (µg/ml)	Residual FV activity Time for which protease incubated with FV (min)		
	0	10	20
10	93	91	100
30	100	93	28
100	100	29	13

[0077] This example demonstrates that FV was inactivated by the protease over time.

Example 6

[0078] The influence of the "FVII activator" on clotting times in so-called global tests was investigated using Schnitger and Gross coagulometers. All the difference values listed correspond to the clotting times which were shortened by this amount.

NAPTT (non-activated partial thromboplastin time)

[0079] The protease-containing solution was diluted with buffer down to 100, 30, 10 and 3 μg/ml. 100 μl of each of these solutions were incubated, at 37°C for 2 min, with 100 μl of citrate plasma (standard human plasma pool or individual donors) and 100 μl of Pathromtin®, after which 100 μl of 25 mM $CaCl_2$ were added; the clotting times were then determined. The differences between these measured values and the corresponding clotting times obtained with buffer solution instead of the protease were determined.

Sample No.	Clotting time differences (buffer minus sample) (sec) Protease concentration (μg/ml)				
	0	3	10	30	100
Standard human plasma (213 sec)	0	13	20	42	43
1	0	20	33	42	41
2	0	27	31	45	47
3	0	13	14	23	29
4	0	18	37	51	50
5	0	25	49	54	46

[0080] The addition of FVII-acitvator resulted in a concentration dependent shortening of NAPTT.

Plasma recalcification time

[0081] The protease-containing solution was diluted with buffer down to 100, 30, 10 and 3 μg/ml. 100 μl of each of these solutions were incubated with 100 μl of citrate plasma (standard human plasma pool or individual donors) at 37°C for 1 min, after which 100 μl of 25 mM $CaCl_2$ were added; the clotting times were then determined. The differences between these measured values and the corresponding clotting times obtained with buffer solution instead of protease were determined.

Sample No.	Clotting time differences (buffer minus sample) (sec) Protease concentration (μg/ml)				
	0	3	10	30	100
Standard human plasma (283 sec)	0	17.2	15.1	30.5	50.4
1	0	29.8	51.7	60.3	90.1
2	0	25.2	51.7	69.5	101.3
3	0	28.0	—	39.0	74.6
4	0	27.3	42.7	55.6	91.8
5	0	44.3	69.1	101.2	134.2

PT (prothombin time)

[0082] The protease-containing solution was diluted with buffer down to 100, 30, 10 and 3 μg/ml. 100 μl of each of these solutions were incubated with 100 μl of citrate plasma (standard human plasma pool or individual donors) at 37°C for 1 min, after which 200 μl of Thromborel S® (Dade Behring GmbH) were added; the clotting times were then determined.

[0083] The differences between these measured values and the corresponding clotting times obtained with buffer solution instead of protease were determined.

Sample No.	Clotting time differences (buffer minus sample) (sec) Protease concentration (μg/ml)				
	0	3	10	30	100
Standard human plasma (13.6 sec)	0	1.0	1.7	1.5	2.4
1	0	0.7	1.3	2.4	2.7
2	0	0.3	0.4	1.7	3.1
3	0	0.4	0.7	1.5	1.8
4	0	0.1	0.7	1.8	3.1
5	0	0.3	0.5	1.2	2.8

[0084] The clotting times in the above global tests were shortened in a manner which was dependent on the concentration of the protease. In a corresponding manner, it was possible, after "calibrating" a plasma which was used with a known quantity of the "FVII activator", to determine the protease concentration in a sample by reading off from a standard curve.

Example 7

[0085] The plasminogen activator-activating properties of the "FVII activator" were investigated using single chain urokinase (scuPA) and single chain tPA (sctPA).

Assay:

[0086]

0.1 ml of	PA solution (20 μg of scuPA/ml or 100 μg of sctPA/ml)
+ 0.1 ml of	test buffer or 100 U of heparin/ml in test buffer or 20 mM $CaCl_2$ in the test buffer
+ 0.5 ml of	test buffer
+0.1 ml of	protease/sample (increasing concentrations: 2-10 μg of scuPA/ml or 50-200 μg of sctPA/ml)

Incubation at 37°C +0.1 ml of 100 KIU of aprotinin/ml in test buffer
Incubation at 37°C for 2 min + 0.1 ml ofsubstrate S-2444 (3 mM)

[0087] As a control, aprotinin was introduced initially, instead of the plasminogen activator (PA), prior to the first incubation, and carried through in each case. In return, PA was not added until later, in place of the aprotinin.
[0088] The Difference of the measurements (Δ) ΔOD_{405nm} was determined photometrically. The control values which were obtained were subtracted from the sample/protease values and in this way the PA activity which was caused by the PAA activity was determined (in mIU/min).

Results:

scuPA activation (20 μg of scuPA/ml, 2-10 μg of "FVII activator"/ml)

A. Stimulant: none

[0089]

Incubation time (min)	Resulting PA activity (Δ mIU/min) "FVII activator" (μg/ml)		
	2	5	10
2	25	60	117
5	79	179	165
10	186	449	517

B. Stimulant: heparin

[0090]

Incubation time (min)	Resulting PA activity (Δ mIU/min) "FVII activator" (μg/ml)		
	2	5	10
2	190	332	425
5	330	455	458
10	417	462	460

C. Stimulant: $CaCl_2$

[0091]

Incubation time (min)	Resulting PA activity (Δ mIU/min) "FVII activator" (μg/ml)		
	2	5	10
2	255	370	401
5	338	424	438
10	416	445	448

[0092] The tables illustrate the fact that scuPA was activated in a manner which was dependent on the concentration of the "FVII activator" and on the length of the incubation. At the same time, both heparin and calcium had a stimulatory effect on the activation of the PA which was brought about by the protease.

sctPA activation(100 μg of sctPA/ml, 50-200 μg of "FVII activator"/ml)

[0093] Since the turnover rate of the activated tPA only increases by a factor of 3-4 as compared with the tPA proenzyme (while that of uPA increases by a factor of 1000-1500), higher concentrations of the two coreactants (see above) had to be selected in order to obtain an analyzable measurement signal.

Incubation time (min)	Resulting PA activity (Δ mIU/min) "FVII activator" (200 μg/ml)
1	10.2
2	16.8
5	38.8
10	60.2
20	73.3

B. Dependence on the concentration of the "FVII activator" (incubation time: 20 min, at 37°C), stimulant: heparin (100 IU/ml)

[0094]

"FVII activator" (μg/ml)	PA activity (Δ mIU/min)
50	33.6
100	51.0
200	71.9

C. Stimulants (period of incubation: 20 min, at 37°C)

[0095]

Stimulant	PA activity (Δ mIU/min)
None	5.9
$CaCl_2$	25.3

Table continued

Stimulant	PA activity (Δ mIU/min)
Heparin	63.8

[0096] The tables demonstrate that sctPA was also activated in a manner which was dependent on the concentration of the protease and on the incubation time. Both heparin and calcium ions had a stimulatory effect on the PA-activating ability of the "FVII activator".

Example 8

[0097] Two FVIII-containing solutions, one of which was essentially free of von Willebrand Factor while the other contained vWF, were incubated with the abovementioned protease in the presence of calcium. After predetermined times, the residual FVIII activities were determined by means of a chromogenic test and related to the control assays without protease.

[0098] For this, 25 μl of a solution containing 0.1 IU of FVIII/ml were treated with the same volume of the protease solution (10 μg/ml) and the whole was mixed with 25 μl of $CaCl_2$ (25 mM). After incubation periods of 0, 5, 10 and 20 min at 37°C, the assays were in each case treated with 400 μl of a solution containing 200 KIU of aprotinin/ml in order to stop the proteolytic activity of the protease. Preliminary experiments had shown that this concentration of aprotinin had no significant interfering effect on the FVIII activity test described below (assays 1 + 3). In assay 2, the protease was incubated with aprotinin prior to contact with FVIII, after which the procedure was as described above.

[0099] In each case, 50 μl of the stopped sample (or after further dilution) were then treated with the so-called factor reagent, essentially composed of FIXa, FX and a thrombin inhibitor, and incubated at 37°C for 10 min. Following the addition of 50 μl of a chromogenic substrate which is cleaved by activated FX, the reaction was stopped after 5 minutes of incubation by adding 50 μl of acetic acid (50%); the ΔOD_{405nm} was then measured. The FVIII activity (mIU) was ascertained with the aid of a standard curve which was constructed using a dilution series which was prepared from the FVIII concentrate and which was included in the test.

[0100] The FVIII activities are given in percentages of the controls to which protease was not added.

Results:

[0101]

Assay	FVIII activity (%) Incubation period (min)			
	0	5	10	20
1. FVIII	97	27	11	<1
2. FVIII/aprotinin	98	97	97	96
3.FVIII/vWF	98	16	14	1

[0102] In the presence of $CaCl_2$ (in this case 6.25 mM), FVIII was inactivated by the protease in a manner which was dependent on the length of the incubation. The vWF did not protect the FVIII from inactivation by the protease. Inhibition of the protease with aprotinin prior to contact with FVIII prevented the latter from being inactivated.

Example 9

[0103] This experimental series was carried out as described in example 1/assay 1, but in this case the concentrations of calcium in the mixtures of protease and FVIII were varied. For this, $CaCl_2$ was added, from the stock solution of calcium, up to the final concentrations shown in Figure 3.

Results:

[0104] If the concentration of calcium in the assay is decreased below 1 mM, approx. 50% of the FVIII is then spared under these conditions. Below 0.5 mM calcium, the percentage spared is more than 60% (Fig. 1).

Example 10

[0105] The influence of the "FVII activator" on the clotting times in so-called global tests was investigated by means of thromboelastography.

[0106] The change in the shear elasticity or the strength of the relevant blood clot was recorded continuously using a Hilgard TEG meter (from Hellige). The so-called r and k values are, respectively, the times from the beginning of blood withdrawal and from the start of the clotting reaction, and, in the case of citrate blood plasma, the time of recalcification until the TEG curve has been broadened by 1 mm and the time from the endpoint of the r value until the curve has been broadened to 20 mm (clot formation time).

[0107] For this, aliquots of 150 µl of blood or plasma from 5 donors were in each case incubated in the measuring cuvettes at 37°C for 2 min, after which 50 µl of sample (protease) were mixed in. The reaction was started by adding 100 µl of 25 mM $CaCl_2$. The final concentration of the "FVII activator" in the assay was 15 µg/ml. The shortening of the r time was measured in relation to the assay which contained buffer instead of the sample.

Results:

[0108]

Blood No.	Sample	r time (min)	k time (min)	r+k time (min)
1	Protease	5.2	3.4	8.6
1	Buffer	7.8	5.6	13.4
2	Protease	5.2	5.1	10.3
2	Buffer	6.8	7.1	13.9
3	Protease	4.0	5.2	9.2
3	Buffer	6.5	6.3	12.8
4	Protease	4.5	4.8	9.3
4	Buffer	4.8	6.0	10.8
5	Protease	4.2	3.8	8.0
5	Buffer	7.0	5.8	12.8

Plasma No.	Sample	r time (min)
1	Protease	9.0
1	Buffer	11.3
2	Protease	9.2
2	Buffer	12.5
3	Protease	9.5
3	Buffer	9.6
4	Protease	8.2
4	Buffer	12.1
5	Protease	9.7
5	Buffer	14.1

[0109] This example makes clear that, in almost all cases, addition of the protease resulted in a marked shortening of the clotting time. In this present instance, the fibrinolytic properties of the "FVII activator" receded into the background. A reason for this is that in "normal subjects", the concentrations of plasminogen activator in the plasma lie in the nanogram region and do not have any effect in the in-vitro clotting test.

Example 11

[0110] The FVIII-bypassing activity of the protease was demonstrated by the following experimental assay: thromboelastography was used as the measuring technique. The r time was evaluated (see Example 10). A sample of whole blood was incubated with a monoclonal antibody, whose FVIII-activity-inhibiting properties were known, in order to simulate the presence of a naturally occurring FVIII inhibitor (antibody against FVIII). This sample was compared with the whole blood sample control (buffer instead of Mab). The FEIB activity of the protease was tested by adding the

protease (final concentration 17 μg/ml) to the whole blood sample which had been inhibited by the Mab. Protease was added to a further sample, and the effect of the protease, on its own, on the r time was determined.

Results:

[0111]

	r time
Whole blood control	8.0
Whole blood + mAb	11.0
Whole blood + mAb + protease	8.0
Whole blood + protease	3.5

[0112] The lengthening of the r time, caused by the anti-FVIII mAb, was normalized once again by the presence of the protease, thereby illustrating the FEIB activity of the protease. On its own, the protease shortened the clotting time, as already demonstrated above.

Example 12

[0113] The following substances were added to a solution, which contained 50 μg of the FVII-activating protease/ ml, to give the corresponding final concentrations:

25 mM Na citrate
25 mM HEPES
100 mM arginine
0.75 g of sucrose/ml

[0114] The solution was divided into portions and the aliquots were in each case adjusted to different pH values of from 5.0 to 8.6 and then heated at 60°C for 10 hours.

[0115] The activities of the heated protease solutions were determined in a chromogenic test, with the time-dependent amidolysis of the chromogenic substrate S2288 (H-D-Ile-Pro-Arg-pHA x 2 HCl, Chromogenix AB, Sweden) being recorded. This activity was expressed as a percentage of the aliquots which were unheated and were measured in parallel:

Results:

[0116]

Assay	Activity (%)
Starting material	100
pH 5.0	76
pH 5.5	65
pH 6.1	81
pH 6.5	50
pH 7.1	43
pH 7.5	46
pH 8.1	46
pH 8.6	32

[0117] This series of experiments makes clear that the stabilization, particularly in the acid pH range, has markedly reduced the inactivation of the protease. The slight "breakthrough" at pH 5.5 can be explained by the fact that the isoelectric point of the protease is in this range. Na citrate prevents a loss of activity of > 50% occurring in the preferred pH range.

Example 13

[0118] The assay at pH 6.1 (Example 1) showed the best stabilization of the protease. Accordingly, different additives were tested at pH 6.0 and evaluated as described in Example 1:

The following final concentrations were set, with the concentration of the protease being 50 μg/ml:

50 mM Na citrate/50 mM NaCl, pH 6.0
0.75 g of sucrose/ml
100 mM glycine
100 mM arginine

Results:

[0119]

Assay	Activity (%)
Starting material	100
Na citrate/NaCl	54
Na citrate/NaCl/sucrose	85
Na citrate/NaCl/sucrose/glycine	92
Na citrate/NaCl/sucrose/arginine	97

[0120] Marked stabilization of the protease was demonstrated by adding sucrose and in each case one amino acid.

Claims

1. A test system for the qualitative and quantitative detection of the FVII-activating protease, which

a) is inhibited by the presence of aprotinin,
b) is increased in its activity by calcium ions and/or heparin or heparin-related substances and
c) in SDS-PAGE, on subsequent staining in the non-reduced state, has one or more bands in the molecular weight range from 50 to 75 kDa and in the reduced state has a band at 40 to 55 kDa and one or more bands in the molecular weight range from 10 to 35 kDa and wherein
d) the band obtained in SDS-PAGE in the reduced state in the molecular weight range from 60 to 65 kDa and from 40 to 55 kDa has an amino acid sequence of LLESLDP and the band obtained in the molecular weight range from 10 to 35 kDa has an amino acid sequence of IYGGFKSTAGK,

or its proenzyme, wherein the protease is measured by means of

a) its activity inactivating the blood clotting factors VIII/VIIIa or V/Va or
b) its activity reducing the blood clotting times in global clotting tests or
c) its activity activating plasminogen activators or
d) its activity activating FVII

and wherein the unknown FVII-activating protease concentration is determined by comparison with a standard curve of increasing quantities of FVII-activating protease

2. The test system as claimed in claim 1, wherein the activity reducing the blood clotting times of the FVII-activating protease is determined by means of the

a) non-activated partial thromboplastin time (NAPTT) or of the
b) prothrombin time (PT) or of the
c) plasma recalcification time or of the

d) activated partial thromboplastin time (APTT) and

and wherein the unknown FVII-activating protease concentration is determined by comparison with a standard curve of increasing quantities of FVII-activating protease.

3. The test system as claimed in claim 1, wherein the activity of the FVII-activating protease activating and/or potentiating the plasminogen activators is measured by the activation of the

   a) single chain urokinase PA (scuPA, single chain urokinase plasminogen activator) or of the
   b) single chain tPA (sctPA, single chain tissue plasminogen activator)

   and wherein the unknown FVII-activating protease concentration is determined by comparison with a standard curve of increasing quantities of FVII-activating protease.

4. The test system as claimed in claims 1 to 3, which contains calcium ions in an amount of more than 0.001 mM, preferably in an amount of more than 0.005 mM.

5. A test system wherein the FVII-activating protease as **characterized in** claim 1 and/or a mixture of its proenzyme and appropriate proenzyme activators are used to test the prothrombin time substituting tissue factor/thromboplastin.

6. A test system where the FVII-activating protease as **characterized in** claim 1 and/or a mixture of its proenzyme and appropriate proenzyme activators are used to test the functionality of plasminogen activators and for quantification of the single chain plasminogen activator forms.

7. The test system as claimed in claims 1, 3, 4 and 6, wherein the activity potentiating the plasminogen activators

   a) is measured using a chromogenic test or
   b) in a coupled reaction in the presence of plasminogen, the plasmin formation itself or the dissolution of a fibrin clot brought about by plasmin being determined,
   and wherein if FVII-activating protease as **characterized in** claim 1 is measured the unknown FVII-activating protease concentration is determined by comparison with a standard curve of increasing quantities of FVII-activating protease.

8. A test system wherein the presence of FVII can be determined qualitatively and quantitatively by adding FVII-activating protease as **characterized in** claim 1.

9. The FVII-activating protease as **characterized in** claim 1 or its proenzyme for use in therapy.

10. A pharmaceutical preparation, which contains an amount of the FVII-activating protease as **characterized in** claim 1 and/or its proenzyme adequate for the dissolution of fibrin-containing thrombi.

11. The pharmaceutical preparation as claimed in claim 10, which, apart from the FVII-activating protease as **characterized in** claim 1 and/or its proenzyme, contains single chain or two chain plasminogen activators (PA) and/or anticoagulants.

12. The pharmaceutical preparation as claimed in claims 10 and 11, which additionally contains soluble calcium salts and/or heparin or heparan-sulfate or dextran-sulfate.

13. A process for obtaining the FVII-activating protease as **characterized in** claim 1 or its proenzyme, which comprises obtaining it from blood plasma or prothrombin (PPSB) concentrates after prior anion exchange chromatography by means of affinity chromatography using heparin or heparan sulfate or dextran sulfate.

14. A process for removing the FVII-activating protease as **characterized in** claim 1 or its proenzyme, which comprises removing it from blood plasma or other FVIII- or FV-containing solutions after prior anion exchange chromatography by means of affinity chromatography using heparin or heparan sulfate or dextran sulfate.

15. A process for the pasteurization of a pharmaceutical preparation comprising the FVII-activating protease as **characterized in** claim 1 and/or its proenzyme, which process comprises preparing the preparation

a) in a pH range from 3.5 to 8.0
b) with addition of one or more amino acids in an amount of > 0.01 mol/l and/or
c) with addition of a sugar or a combination of a number of sugars in a total amount of > 0.05 g/ml and/or
d) with addition of one or more substances which are able to complex calcium ions under pasteurization conditions.

16. A reagent which contains the FVII-activating protease as **characterized in** claim 1 together with FVII-activating protease activity enhancing compounds or its proenzyme together with activators of the proenzyme.

17. A reagent as claimed in claim 16 which contains the FVII-activating protease or its proenzyme together with calcium ions, heparin, heparan sulfate or dextran sulfate.

18. The use of a reagent as claimed in claim 17 for the detection of factor VII.

19. The use of the FVII-activating protease as **characterized in** claim 1 or its proenzyme, optionally together with proenzyme activators prepared from blood plasma or prothrombin complex (PPSB) concentrates for the preparation of

- a medicament for the promotion of wound healing and hemostasis,
- an additive of a fibrin adhesive or fleece or other release system which is suitable for rapid wound closure, based on fibrin,
- a medicament for substitution in inborn or acquired deficiency states of this protease or its proenzyme, in the presence of antibodies against the blood clotting factor VIII or
- a reagent for the in vitro activation of factor VII.

20. Use of FVII-activating protease as **characterized in** claim 1 for in vitro diagnostic methods in order to

a) qualitatively or quantitatively determine FVII-activating protease
and/or
b) qualitatively or quantitatively determine Factor VII
and/or
c) test the functionality of plasminogen activators and for quantification of the single chain plasminogen activator forms
and/or
d) test the prothrombin time substituting tissue factor/thromboplastin.

Revendications

1. Système test pour la détection qualitative et quantitative de la protéase activant FVII, qui

a) est inhibée par la présence d'aprotinine,
b) voit son activité accrue par les ions calcium et/ou l'héparine ou les substances en rapport avec l'héparine et
c) dans une SDS-PAGE, lors d'une coloration conséquente à l'état non réduit, a une ou plusieurs bandes dans la gamme de poids moléculaire compris entre 50 et 75 kDa et à l'état réduit a une bande à 40 à 55 kDa et une ou plusieurs bandes dans la gamme de poids moléculaire de 10 à 35 kDa et dans lequel
d) la bande obtenue dans la SDS-PAGE à l'état réduit dans la gamme de poids moléculaire de 60 à 65 kDa et de 40 à 55 kDa a une séquence d'acides aminés de LLESLDP et la bande obtenue dans la gamme de poids moléculaire de 10 à 35 kDa a une séquence d'acides aminés de IYGGFKSTAGK,

ou sa proenzyme, dans lequel la protéase est mesurée au moyen de :

a) son activité inactivant les facteurs de coagulation sanguine VIII/VIIIa ou V/Va ou
b) son activité réduisant le temps de coagulation sanguine dans les tests de coagulation globaux ou
c) son activité activant les activateurs du plasminogène ou
d) son activité activant FVII

et dans lequel la concentration de la protéase activant FVII inconnue est déterminée par comparaison avec une

courbe standard de quantité croissante de protéase activant FVII.

2. Système test selon la revendication 1, dans lequel l'activité réduisant les temps de coagulation sanguine de la protéase activant FVII est déterminée au moyen :

   a) du temps de céphaline non activé (NAPTT) ou du
   b) temps de Quick (PT) ou du
   c) temps de Howell ou du
   d) temps de céphaline activé (APTT) et

   dans lequel la concentration de la protéase activant FVII inconnue est déterminée par comparaison avec une courbe standard des quantités croissantes de protéase activant FVII.

3. Système test selon la revendication 1, dans lequel l'activité de la protéase activant FVII activant et/ou potentialisant les activateurs du plasminogène est mesurée par l'activation de :

   a) un PA urokinase monocaténaire (scuPA, activateur du plasminogène urokinase monocaténaire) ou du
   b) tPA monocaténaire (sctPA, activateur du plasminogène tissulaire monocaténaire)

   et dans lequel la concentration de protéase activant FVII inconnue est déterminée par comparaison avec une courbe standard des quantités croissantes de protéase activant FVII.

4. Système test selon les revendications 1 à 3, qui contient des ions calcium en une quantité de plus de 0,001 mM, de préférence en une quantité de plus de 0,005 mM.

5. Système test dans lequel la protéase activant FVII telle que **caractérisée** dans la revendication 1 et/ou un mélange de sa proenzyme et des activateurs proenzymatiques appropriés sont utilisés pour tester le facteur tissulaire substituant le temps de Quick/thromboplastine.

6. Système test où la protéase activant FVII telle que **caractérisée** dans la revendication 1 et/ou un mélange de sa proenzyme et des activateurs proenzymatiques appropriés sont utilisés pour tester la fonctionnalité des activateurs du plasminogène et, pour la quantification des formes d'activateurs du plasminogène monocaténaires.

7. Système test selon les revendications 1, 3, 4 et 6, dans lequel l'activité potentialisant les activateurs du plasminogène

   a) est mesurée en utilisant un test chromogènique ou
   b) dans une réaction couplée en présence du plasminogène, la formation de plasmine elle-même ou la dissolution d'un caillot fibrineux provoqué par plasmine étant déterminée

   et dans lequel si la protéase activant FVII telle que **caractérisée** dans la revendication 1 est mesurée, la concentration en protéase activant FVII inconnue est déterminée par comparaison avec une courbe standard des quantités croissantes de protéase activant FVII.

8. Système test dans lequel la présence de FVII peut être déterminée qualitativement et quantitativement en ajoutant une protéase activant FVII telle que **caractérisée** dans la revendication 1.

9. Protéase activant FVII telle que **caractérisée** dans la revendication 1 ou sa proenzyme pour une utilisation en thérapie.

10. Préparation pharmaceutique, qui contient une quantité de la protéase activant FVII telle que **caractérisée** dans la revendication 1 et/ou sa proenzyme adéquate pour la dissolution de thrombi contenant de la fibrine.

11. Préparation pharmaceutique selon la revendication 10, qui, à l'exception de la protéase activant FVII telle que **caractérisée** dans la revendication 1 et/ou sa proenzyme, contient des activateurs du plasminogène monocaténaires ou bicaténaires (PA) et/ou des anticoagulants.

12. Préparation pharmaceutique selon les revendications 10 et 11, qui contiennent de plus des sels de calcium solubles et/ou de l'héparine ou de l'héparane-sulfate ou du dextrane-sulfate.

13. Processus pour obtenir la protéase activant FVII telle que **caractérisée** dans la revendication 1 ou sa proenzyme, qui comprend l'obtention de celle-ci à partir du plasma sanguin ou de concentrés prothrombine (PPSB) après chromatographie d'échange anionique préalable au moyen d'une chromatographie d'affinité en utilisant de l'héparine ou de l'héparane-sulfate ou du dextrane-sulfate.

14. Processus de retrait de la protéase activant FVII tel que **caractérisé** dans la revendication 1 ou sa proenzyme, qui comprend son retrait du plasma sanguin ou d'autres solutions contenant FVIII ou FV après chromatographie d'échange anionique préalable au moyen d'une chromatographie d'affinité en utilisant de l'héparine ou de l'héparane-sulfate ou du dextrane-sulfate.

15. Processus pour la pasteurisation d'une préparation pharmaceutique comprenant la protéase activant FVII telle que **caractérisée** dans la revendication 1 et/ou sa proenzyme, lequel processus préparant la préparation :

    a) dans une gamme de pH de 3,5 à 8,0
    b) avec ajout d'un ou plusieurs acides aminés en une quantité supérieure à 0,01 mol/l et/ou
    c) avec ajout d'un sucre ou d'une combinaison d'un certain nombre de sucres dans une quantité totale supérieure à 0,05 g/ml et/ou
    d) avec ajout d'une ou plusieurs substances qui sont capables de complexer des ions calcium dans des conditions de pasteurisation.

16. Réactif qui contient la protéase activant FVII telle que **caractérisée** dans la revendication 1 conjointement avec des composés stimulant l'activité de la protéase activant FVII ou sa proenzyme conjointement avec des activateurs de la proenzyme.

17. Réactif selon la revendication 16 qui contient la protéase activant FVII ou sa proenzyme conjointement à des ions calcium, de l'héparine, de l'héparane-sulfate ou du dextrane-sulfate.

18. Utilisation d'un réactif selon la revendication 17 pour la détection d'un facteur VII.

19. Utilisation de la protéase activant FVII telle que **caractérisée** dans la revendication 1 ou sa proenzyme, éventuellement conjointement à des activateurs proenzymatiques préparés à partir de plasma sanguin ou de concentrés d'un complexe prothrombique (PPSB) pour la préparation de

    - d'un médicament pour la favorisation d'une cicatrisation de lésion et une hémostase,
    - d'un additif d'un adhésif de fibrine ou une compresse ou autre système de libération qui est approprié pour une fermeture de lésion rapide, à base de fibrine,
    - d'un médicament pour la substitution dans les états de déficience innée ou acquise de cette protéase ou de sa proenzyme, en présence d'anticorps anti-facteur VIII de coagulation sanguine ou
    - d'un réactif pour l'activation in vitro du facteur VII.

20. Utilisation d'une protéase activant FVII telle que **caractérisée** dans la revendication 1 pour des procédés de diagnostic in vitro afin de :

    a) déterminer qualitativement ou quantitativement la protéase activant FVII
    et/ou
    b) déterminer qualitativement ou quantitativement le facteur VII
    et/ou
    c) tester la fonctionnalité des activateurs du plasminogène et pour la quantification des formes d'activateurs de plasminogène monocaténaires
    et/ou
    d) tester le facteur tissulaire substituant le temps de Quick/thromboplastine.

Patentansprüche

1. Testsystem für die qualitative und quantitative Detektion der FVII-aktivierenden Protease, welche

    a) durch das Vorhandensein von Aprotinin inhibiert wird,

b) in ihrer Aktivität durch Calciumionen und/oder Heparin oder Heparin-verwandte Substanzen verstärkt wird, und

c) in SDS-PAGE, bei der darauffolgenden Anfärbung des nicht-reduzierten Zustandes, eine oder mehrere Banden im Molekulargewichtsbereich von 50 bis 75 kDa besitzt und im reduzierten Zustand eine Bande bei 40 bis 55 kDa und eine oder mehrere Banden im Molekulargewichtsbereich von 10 bis 35 kDa aufweist, und worin

d) die im SDS-PAGE im reduzierten Zustand im Molekulargewichtsbereich von 60 bis 65 kDa und von 40 bis 55 kDa erhaltene Bande eine Aminosäuresequenz von LLESLDP aufweist and die im im Molekulargewichtsbereich von 10 bis 35 kDa erhaltene Bande eine Aminosäuresequenz von JYGGFKSTAGK besitzt,

oder von derem Proenzym, wobei die Protease mittels

a) ihrer Aktivität bei der Inaktivierung der Blutgerinnungsfaktoren VIII/VIIIa oder V/Va oder
b) ihrer Aktivität bei der Reduzierung der Blutgerinnungszeiten im Gesamtblutgerinnungstest oder
c) ihrer Aktivität bei der Aktivierung von Plasminogenaktivatoren oder
d) ihrer Aktivität bei der Aktivierung von FVII

gemessen wird, und worin die unbekannte Konzentration der FVII-aktivierenden Protease mittels Vergleich mit einer Standardkurve von ansteigenden Mengen an FVII-aktivierender Protease ermittelt wird.

2. Testsystem nach Anspruch 1, worin die Aktivität bei der Verringerung der Blutgerinnungszeiten der FVTI-aktivierenden Protease mittels

a) der nicht-aktivierten Partialthromboplastinzeit (NAPTT) oder
b) der Prothrombinzeit (PT) oder
c) der Plasmarekalzifizierungszeit oder
d) der aktivierten Partialthromboplastinzeit (APTT)

bestimmt wird, und worin die unbekannte Konzentration der FVII-aktivierenden Protease durch Vergleich mit einer Standardkurve mit ansteigenden Mengen an FVII-aktivierender Protease ermittelt wird.

3. Testsystem nach Anspruch 1, worin die Aktivität der FVII-aktivierenden Protease bei der Aktivierung und/oder der Potenzierung der Plasminogenaktivatoren durch die Aktivierung

a) des Einzelkettenurokinase-PA (scuPA, Einzelkettenurokinaseplasminogenaktivator) oder
b) des Einzelketten-tPA (sctPA, Binzelkettengewebeplasminogenaktivator)

gemessen wird, und worin die unbekannte Konzentration an FVII-aktivierender Protease durch Vergleich mit einer Standardkurve mit ansteigenden Mengen von FVII-aktivierender Protease ermittelt wird.

4. Testsystem nach den Ansprüchen 1 bis 3, welches Calciumionen in einer Menge von Mehr als 0,001 mM, vorzugsweise in einer Menge von mehr als 0,005 mM enthält.

5. Testsystem, worin die FVII-aktivierende Protease, wie sie in Anspruch 1 **gekennzeichnet** ist, und/oder ein Gemisch aus derem Proenzym und geeigneten Proenzymaktivatoren verwandet wird, um die Gewebefaktor/Thromboplastin substituierende Prothrombinzeit zu testen.

6. Testsystem, worin die FVIT-aktivierende Protease, wie sie in Anspruch 1 **gekennzeichnet** ist, und/oder ein Gemisch von derem Proenzym und geeigneten Proenzymaktivatoren verwendet wird, um die Funktionalität von Plasminogenaktivatoren zu testen and zur Quantifizierung der Binzelkettenplasminogenaktivatorformen.

7. Testsystem nach den Ansprüchen 1, 3, 4 und 6, worin die Aktivität bei der Potenzierung der Plasminogenaktivatoren

a) unter Verwendung eines chromogenen Tests oder
b} in einer gekoppelten Reaktion in Gegenwart von Plasminogen, der Plasminausbildung selbst oder der Auflösung eines Fibringerinnsels, verursacht durch das zu ermittelnde PlasMin,

gemessen wird, und worin bei Messung der FVII-aktivierenden Protease, wie sie in Anspruch 1 **gekennzeichnet** ist, die unbekannte Konzentration der FVII-aktivierenden Protease durch Vergleich mit einer Standerdkurve mit

ansteigenden Mengen an FVII-aktivierender Protease ermittelt wird.

8. Testsystem, worin das Vorhandensein von FVII qualitativ und quantitativ durch Zusetzen von FVII-aktivierender Protease, wie sie in Anspruch 1 **gekennzeichnet** ist, ermittelt werden kann.

9. Die FVII-aktivierende Protease, wie sie in Anspruch 1 **gekennzeichnet** ist, oder deren Proenzym zur Verwendung in der Therapie.

10. Pharmazeutische Zubereitung, welche eine Menge der FVII-aktivierenden Protease, wie sie in Anspruch 1 **gekennzeichnet** ist und/oder deren Proenzym enthält, welche für die Auflösung von Fibrin-enthaltenden Thromben adäquat sind.

11. Pharmazeutische Zubereitung nach Anspruch 10, welche abgeseben von der FVII-aktivierenden Protease, wie sie in Anspruch 1 **gekennzeichnet** ist, und/oder cerem Proenzym Einzelketten- oder Doppelkettenplasminogenaktivatoren (PA) und/oder Antikoagulantien enthält.

12. Pharmazeutische Zubereitung nach Anspruch 10 und 11, welche zusätzlich lösliche Calclumsalze und/oder Heparin oder Heparan-Sulfat oder Dextran-Sulfat enthält.

13. Verfahren zur Gewinnung der FVII-aktivierenden Protease, wie sie in Anspruch 1 **gekennzeichnet** ist, oder von derem Proenzym, welches das Gewinnen dieser/dieses aus dem Blutplasma oder aus Prothrombinkenzentraten (PPSB-Konzentraten) nach einer vorhergehenden Anionenaustauscherchromatographie mittels Affinitätschromatographie unter Verwendung von Heparin oder Keparan-Sulfat oder Dextran-Sulfat umfaßt.

14. Verfahren zur Entfernung der FVII-aktivierenden Protease, wie sie in Anspruch 1 **gekennzeichnet** ist, oder von derem Proenzym, welches das Entfernen dieser/dieses aus dem Blutplasma oder anderen FVIII oder FV enthaltenden Lösungen nach einer vorhergehenden Anionenaustauscherchromatographie mittels Affinitätschromatographie unter Verwendung von Heparin oder Heparan-Sulfat oder Dextran-Sulfat umfaßt.

15. Verfahren zur Pasteurisierung einer pharmazeutischen Zubereitung, umfassend die FVII-aktivierende Protease, wie sie in Anspruch 1 **gekennzeichnet** ist, und/oder deren Proenzym, welches Verfahren das Herstellen der Zubereitung

    a) in einem pH-Bereich von 3,5 bis 8,0
    b) unter Zugabe von einer oder mehreren Aminosäuren in einer Menge von >0,01 Mol/l und/oder
    c) unter Zugabe eines Zuckers oder in Kombination mit einer Anzahl von Zuckern in einer Gesamtmenge von >0,05 g/ml und/oder
    d) unter Zugabe von einer oder mehreren Substanzen, welche fähig sind, Calciumionen unter Pasteurisierungsbedingungen zu komplexieren,

    umfaßt.

16. Reagenz, welches die FVII-aktivierende Protease, wie sie in Anspruch 1 **gekennzeichnet** ist, gemeinsam mit die Aktivität von FVII-aktivierender Protease erhöhenden Verbindungen, oder deren Proenzym gemeinsam mit Proenzymaktivatoren enthält.

17. Reagenz nach Anspruch 16, welches die FVII-aktivierende Protease oder deren Proenzym gemeinsam mit Calciumioren, Heparin, Heparan-Sulfat oder Dextran-Sulfat umfaßt.

18. Verwendung eines Reagenzes nach Anspruch 17 zur Detektion von Faktor VII.

19. Verwendung der FVII-aktivierenden Protease, wie sie in Anspruch 1 **gekennzeichnet** ist, oder von derem Proenzym wahlweise gemeinsam mit Proenzymaktivatoren, gewonnen aus Blutplasma oder Prothrombinkomplexkonzentraten (PPSB-Konzentraten), zur Herstellung

    - eines Arzneimittels zur Förderung der Wundheilung und der Hamostasis,
    - eines Additivs eines Fibrinklebstoffs oder eines -vlieses oder eines anderen Freisetzungssystems, basierend auf Fibrin, welches zum raschen Nundverschluß geeignet ist,

- eines Arzneimittels zur Substitution dieser Protease oder von deren Proenzym bei angeborenen oder erworbenen Mangelzuständen in Gegenwart von Antikörpern gegen den Blutgerinnungsfaktor VIII, oder
- eines Reagenzes für die in vitro-Aktivierung von Faktor VII.

20. Verwendung von FVII-aktivierender Protease, wie sie in Anspruch 1 **gekennzeichnet** ist, für in vitro-diagnostische Verfahren,

a) zur qualitativen oder quantitativen Bestimmung der - FVII-aktivierenden Protease
und/oder
b) zur qualitativen oder quantitativen Bestimmung des Faktors VII
und/oder
c) zur Testung der Funktionalität von Plasminogenaktivatoren und zur Quantifizierung der Rinzelkettenplasminogenaktivatorformen
und/oder
d) zur Testung der Gewebefaktor/Thromboplastin substituierenden Prothrombinzeit.

Figure 1

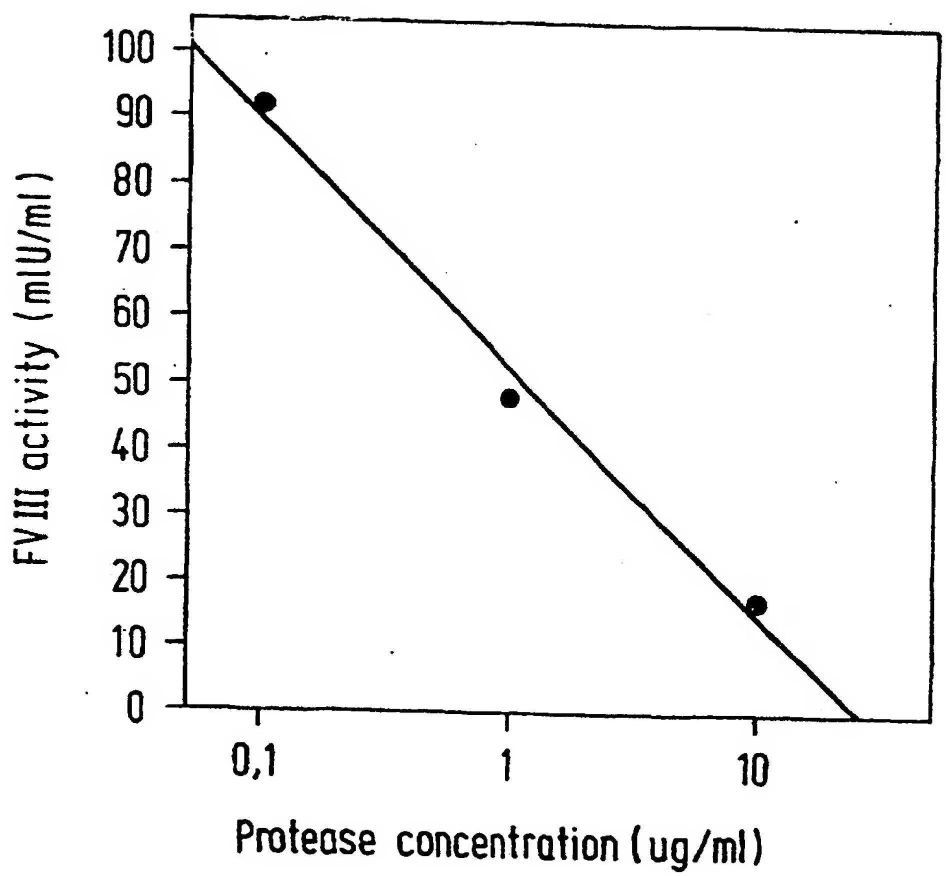

100
90
80
70
60
50
40
30
20
10
0
FVIII activity (mIU/ml)
0,1
1
10
Protease concentration (ug/ml)

Figure 2

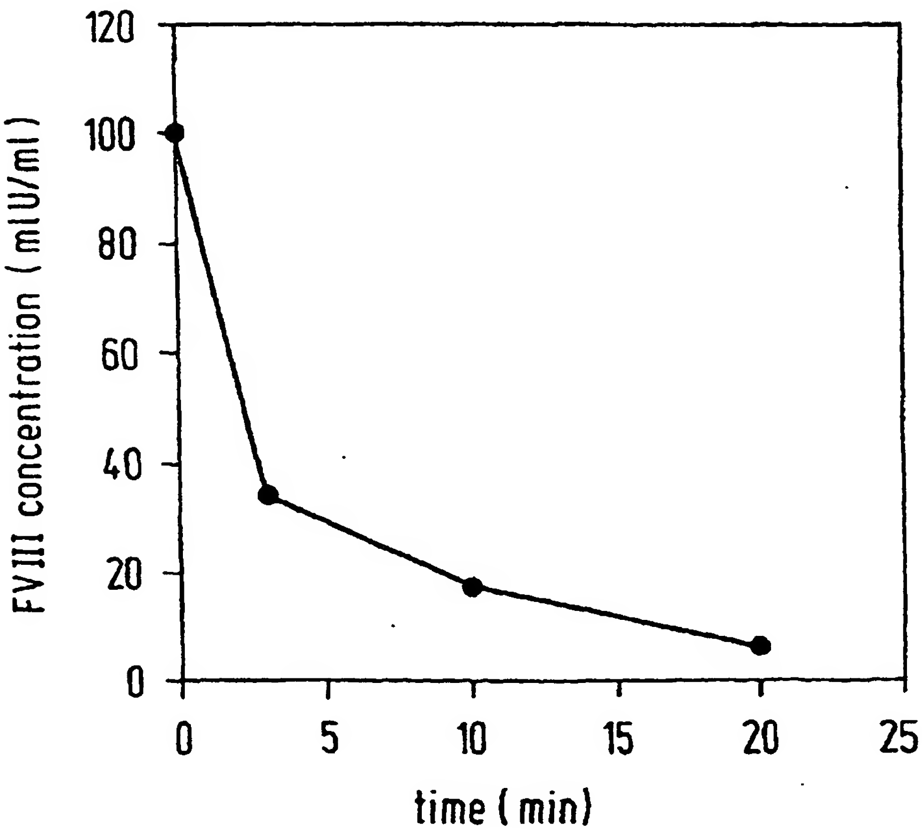
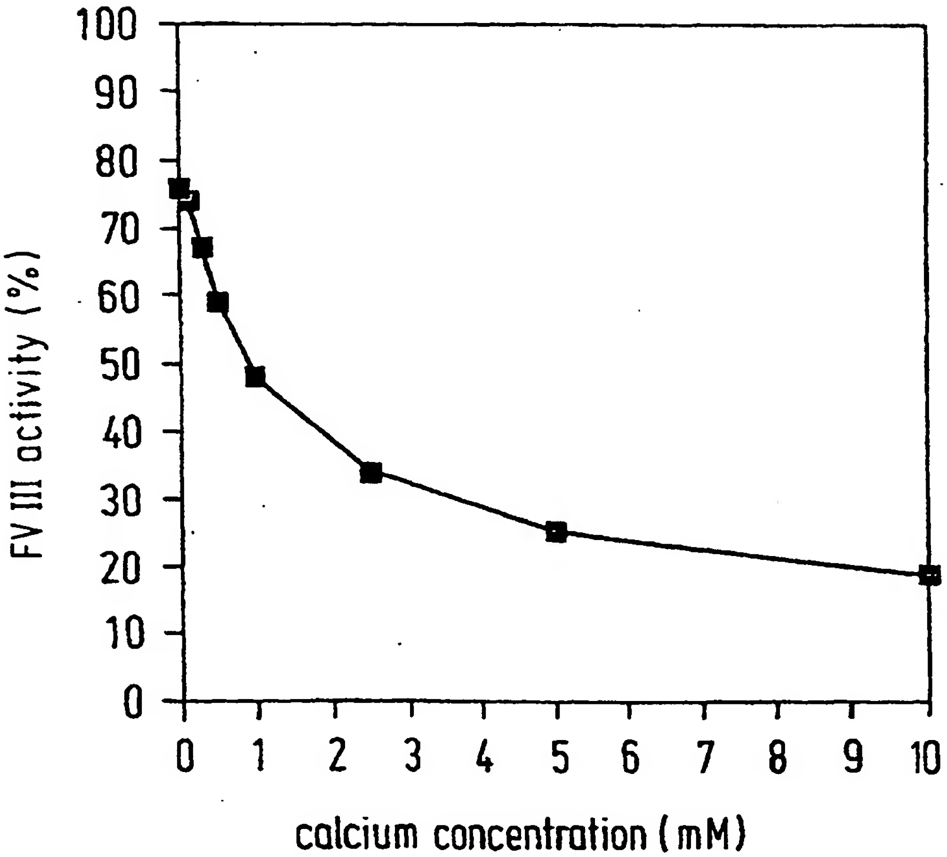

120
100
80
60
40
20
0
FVIII concentration (mIU/ml)
0
5
10
15
20
25
time (min)

Figure 3


FV III activity (%)
100
90
80
70
60
50
40
30
20
10
0
0 1 2 3 4 5 6 7 8 9 10
calcium concentration (mM)